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The Smad protein family has been genetically implicated in transforming growth factor β (TGF-β)-like signaling pathways in *Drosophila* and *Caenorhabditis elegans*. To investigate the role of these proteins in mammalian signaling pathways, we have isolated and studied two murine Smads, Smad1 and Smad5. Using antibodies against Smad1 and Smad5, we show that these two Smads and an immunogenically related protein, presumably also a Smad, are phosphorylated in a time- and dose-dependent manner in response to TGF-β. Bone morphogenetic protein 2, a TGF-β superfamily ligand, induces phosphorylation of only the related Smad protein. Thus, TGF-β superfamily members may use overlapping yet distinct Smads to mediate their intracellular signals. Furthermore, transient overexpression of either Smad1 or Smad5 causes growth arrest, implicating the Smads in growth regulation. This work provides strong biochemical and preliminary functional evidence that Smad1 and Smad5 represent prototypic members of a family of mammalian proteins that may serve as mediators of signaling pathways for TGF-β superfamily members.

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Jonathan Yingling 9-30-96

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A. INTRODUCTION

Breast cancer is the most common cancer in women in the United States. Although endocrine therapy has proven useful in treating approximately one-third of these patients, the tumors inevitably progress to a hormone independent state and no longer respond to these therapies. Thus, it is the goal of this research project to characterize the molecular basis of the TGF-β growth inhibitory in hopes of elucidating additional therapeutic targets.

TGF-βs are a group of multifunctional peptide hormones that play critical roles in many normal cellular processes including the regulation of proliferation, differentiation, extracellular matrix deposition, cell adhesion and migration (11). Perhaps TGF–βs most critical biological activity is its ability to inhibit the growth of a wide variety of cell types including breast epithelia (8). Therefore, loss of components of the TGF–β growth inhibitory pathway in breast epithelia would be expected to contribute to the genesis and progression of breast cancer. Loss of TGF–β responsiveness in MCF-7 cells has been correlated with loss of the type II TGF–β receptor and increased tumorigenesis. Reintroduction of the TGF–β type II receptor in these MCF-7 cells reverses this tumorigenic phenotype indicating the importance of this pathway in preventing breast tumorigenesis (9).

Significant progress has been made over the last 5 years to elucidate the molecular events at the cell membrane involving receptor activation which initiate TGF–βs intracellular signaling pathway. In addition, nuclear events involving induction of cyclin-dependent kinase inhibitors (p15 and p21) have been implicated in mediating TGF–βs growth inhibitory signal (4), (1). However, the nature of the cytoplasmic signaling cascade which transduces the signal from the membrane to the nucleus is just beginning to be resolved. Genetic analyses in *Drosophila* and *C. elegans* have led to the isolation of a variety of components of the TGF–β-like pathway including ligands and receptors which are homologous to those identified in vertebrates. The most recent addition to the list of genetically implicated proteins in *Drosophila* and *C. elegans* is the MAD (*Drosophila*) or SMA (*C. elegans*) family of proteins (7), (6). Mutation in these genes causes phenotypes analogous to those of the TGF–β-like receptors in these organisms thus implicating them in a downstream signaling pathway. Additional evidence from vertebrates is the discovery of a tumor suppressor gene on chromosome 18q which is deleted in ~50% of human pancreatic tumors (2). This gene, called DPC4, is a member of the MAD and SMA family by virtue of it containing the invariant MH1 and MH2 domains at its amino- and carboxy-termini, respectively.

The molecular basis of the role of the MAD family in the TGF–β pathway remains poorly understood. To address this issue we have cloned three murine members of this family termed Smad1, Smad2 and Smad5. We have examined the functional role of two of these proteins, Smad 1 and Smad5, in mediation of the TGF–β pathway in a normal murine mammary epithelial cell line, NMuMg. The results from these studies are summarized in the next section and a manuscript reprint is included in the Appendix.

B. PROGRESS REPORT

In the past twelve months my work has focused on the cloning and characterization of vertebrate members of the MAD family of proteins which have been genetically implicated in TGF−β-like pathways in *Drosophila* and *C. elegans*. Three full length proteins have been cloned from a murine cDNA library, mSmad1, mSmad2 and mSmad5 and antibodies have been generated against mSmad1 and mSmad5. We have utilized these antibodies in a series of experiments in NMuMg and L6 (rat myoblasts) cells to study the involvement of these proteins in TGF−βs growth inhibitory pathway.

Although the level of these proteins is not changed within 24 hours of TGF−β treatment (data not shown), two endogenous Smads are rapidly, but transiently phoshorylated in response to TGF−β (Appendix Fig. 2A). Both antibodies recognize the same two proteins by immunoprecipitation, a 52 kDa and 56 kDa protein. The induced phosphorylation is primarily on serine residues (~90%) with minimal threonine phosphorylation (~10%) as determined by phoshoamino acid analysis (data not shown). This inducible phosphorylation is not specific to NMuMg cells since the same effect is seen in L6 (Appendix Fig. 2C) cells and Mv1Lu cells (data not shown). The dose dependence of this inducible phosphorylation was determined to ensure that the phosphorylation was induced at physiological concentrations of TGF−β (Appendix Fig. 3). TGF−β at 10 pM induced a 3-fold increase in phosphorylation.

TGF−β-like ligands from *Drosophila* and *C. elegans* are most closely related to mammalian bone morphogenetic proteins (BMPs) (3). BMPs have been shown to elicit multiple effects on many different cell types including inhibition of cellular proliferation (3). BMP-2 has been shown to initiate its signaling cascade by binding a heteromeric complex of transmembrane serine/threonine kinase receptors at the cell surface (5). This mechanism is analogous to the TGF−β receptor system (10). However, the similarity between the cytoplasmic pathways that lead to the biological effects of TGF−β and BMP-2 is unknown. The NMuMg cell line is potently inhibited by BMP-2 causing a G1 growth arrest (unpublished results) which afforded us the opportunity to determine if endogenous Smad phosphorylation is induced by BMP-2. Unlike TGF−β, BMP-2 only causes inducible phosphorylation of one endogenous Smad protein (Appendix Fig. 4). However, this protein appears to be identical to one of the proteins which is phosphorylated in response to TGF−β. The possibility that one Smad lies downstream in both the TGF−β and BMP-2 pathways is currently under investigation. The issue of overlapping or distinct Smad proteins downstream of TGF−β superfamily members is currently a source of considerable debate in the field. Our characterization of Smad proteins in NMuMg cells which respond to both TGF−β and BMP-2 should resolve this issue.

To address the functional role of the Smad proteins in TGF−β signaling we cloned mSmad1 and mSmad5 into mammalian expression vectors. Attempts to establish stable cell lines constitutively overexpressing either Smad1 in L6 cells or Smad5 in NMuMg cells were unsuccessful. This result was not unexpected since DPC4 is a tumor suppressor gene and thus suspected to have growth suppressive effects. Consequently we used a modified transient growth arrest assay to assess the ability of Smad1 or Smad5 to

cause a growth arrest in L6 cells. Overexpression of Smad1 or Smad5 causes 30-40% growth inhibition compared to 10% for control transfectants (Appendix Fig. 5). Therefore, Smad1 and Smad5 like DPC4 exhibit growth inhibitory properties, a phenotypic hallmark of TGF−β.

C. CONCLUSIONS

Significant progress has been made in elucidation of a potential downstream signaling pathway involving the Smad family of proteins in TGF–β superfamily signaling pathways. Specifically, two endogenous Smads are phosphorylated in response to TGF–β while only one of these is phosphorylated in response to BMP-2. Furthermore, these proteins when overexpressed in mammalian cells leads to a growth arrest, a phenotypic hallmark of TGF–β. Together with the genetic evidence from *Drosophila* and *C. elegans*, our results establish the Smads as effectors of growth inhibitory signals initiated by TGF–β and BMP-2 in mammalian cells. Our results further suggest that overlapping, yet distinct Smads may be involved in TGF–β superfamily signaling cascades. Future studies will reveal which Smads play a significant role in the pathological process of breast carcinogenesis, analogous to the role of DPC4 in pancreatic carcinogenesis.

D. APPENDIX

Yingling, J.M., Das, P., Savage, C., Zhang, M., Padgett, R.W., and X.F. Wang. 1996. Mammalian dwarfins are phosphorylated in response to transforming growth factor β and are implicated in control of cell growth. Proc Natl Acad Sci USA **93:**8940-4.

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Biochemistry

Mammalian dwarfins are phosphorylated in response to transforming growth factor β and are implicated in control of cell growth

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ABSTRACT **The dwarfin protein family has been genetically implicated in transforming growth factor β (TGF-β)-like signaling pathways in *Drosophila* and *Caenorhabditis elegans*. To investigate the role of these proteins in mammalian signaling pathways, we have isolated and studied two murine dwarfins, dwarfin-A and dwarfin-C. Using antibodies against dwarfin-A and dwarfin-C, we show that these two dwarfins and an immunogenically related protein, presumably also a dwarfin, are phosphorylated in a time- and dose-dependent manner in response to TGF-β. Bone morphogenetic protein 2, a TGF-β superfamily ligand, induces phosphorylation of only the related dwarfin protein. Thus, TGF-β superfamily members may use overlapping yet distinct dwarfins to mediate their intracellular signals. Furthermore, transient overexpression of either dwarfin-A or dwarfin-C causes growth arrest, implicating the dwarfins in growth regulation. This work provides strong biochemical and preliminary functional evidence that dwarfin-A and dwarfin-C represent prototypic members of a family of mammalian proteins that may serve as mediators of signaling pathways for TGF-β superfamily members.**

Transforming growth factor-β (TGF-β) is a multifunctional polypeptide hormone that elicits a wide range of cellular effects, including inhibition of cellular proliferation and transcriptional activation of specific target genes (1, 2). The TGF-β signal is initiated through a heteromeric transmembrane kinase complex of type I and type II receptors (3–6). A potential mechanism of activation for the heteromeric TGF-β receptor complex has been proposed (7). Within the heteromeric complex, the type II receptor phosphorylates the type I receptor, and activation of the type I receptor initiates the intracellular signaling pathway. However, the cytoplasmic signaling pathway(s) that mediate the TGF-β signal are poorly understood. Although a few proteins that interact with the TGF-β receptors have been identified by the yeast two-hybrid system (8–11) and one potential downstream kinase has been implicated in the TGF-β pathway (12), the precise roles for these proteins in TGF-β signaling remain to be elucidated.

Potential insight into the components of the TGF-β signaling pathways has come from the genetic isolation of a novel family of proteins in *Drosophila* and *Caenorhabditis elegans*. Decapentaplegic (dpp) is the TGF-β-like ligand in *Drosophila* (13). Genetic screens for dominant enhancers of a weak *dpp* allele led to the isolation of MAD (Mothers against dpp) (14). Loss-of-function mutations in MAD result in similar phenotypic defects as seen with mutant *dpp* alleles, thus implicating MAD in some aspect of dpp function. *C. elegans* has three MAD homologs, SMA-2, SMA-3, and SMA-4, which have been implicated in the TGF-β-like pathway in the nematode (15). These four genes define a novel family of proteins called dwarfins (15). Mutant alleles of these genes in *C. elegans* give rise to small worms and fused male tail rays. This phenotype is similar to mutant type II receptor (*daf-4*) alleles in *C. elegans* (16), thus implicating the *sma* genes in a pathway downstream of *daf-4* (15). Although the genetic evidence strongly suggests that MAD and the SMA proteins participate in TGF-β superfamily signaling pathways in *Drosophila* and *C. elegans*, the biochemical and functional nature of these proteins remains unknown.

These genetic studies prompted us to investigate the potential role of dwarfins in TGF-β signaling pathways in mammalian cells. A human dwarfin homolog, DPC4, has been identified as a candidate tumor suppressor gene in pancreatic carcinomas (17). Therefore, the dwarfins may play an important role in cellular growth control, including the ability to mediate the growth inhibitory signal initiated by TGF-β or TGF-β superfamily members. We report here the isolation and characterization of two murine dwarfins, dwarfin-A (Dwf-A) and dwarfin-C (Dwf-C). Antibodies against these two proteins reveal that three endogenous dwarfins are inducibly phosphorylated in response to TGF-β, but only one of these is phosphorylated in response to bone morphogenetic protein 2 (BMP-2). This is the first indication that the signals for TGF-β superfamily members may be mediated by overlapping, but distinct, intracellular signaling pathways. Inducible phosphorylation of the dwarfins in mammalian systems provides strong support to the genetic evidence that these proteins are mediating some aspect of TGF-β superfamily signaling pathways. Furthermore, transient overexpression of either Dwf-A or Dwf-C causes a growth arrest, which is consistent with their potential role in mediating TGF-β's growth inhibitory signal.

MATERIALS AND METHODS

Cell Lines. NMuMg (normal murine mammary gland epithelial) and L6 (rat skeletal muscle myoblasts) cells were obtained from the American Type Culture Collection and maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin.

Cloning of Dwarfin cDNAs. A 180-nt fragment of Dwf-A was generated by degenerate PCR (15) and used to screen a 12.5-day mouse embryo library to obtain a partial Dwf-A cDNA of 650 bp. The 650-bp *Eco*RI/*Xho*I clone was radiolabeled with [^{32}P]dCTP using the Prime-It II kit from Stratagene

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Abbreviations: TGF-β, transforming growth factor β; BMP-2, bone morphogenetic protein 2; dpp, decapentaplegic; BrdU, bromodeoxyuridene; DH1 and DH2, dwarfin homology domains 1 and 2; Dwf-A, dwarfin-A; Dwf-C, dwarfin-C.

Data deposition: The sequences reported in this paper have been deposited in the GenBank data base [accession nos. U58992 (dwarfin-A) and U58993 (dwarfin-C)].

‡To whom reprint requests should be addressed.

and used to screen a λgt10 8.5-day mouse embryonic library at low stringency. Briefly, hybridization was at 42°C in 45% formamide, 5× standard saline phosphate/EDTA (SSPE; 0.18 M NaCl/10 mM phosphate, pH 7.4/1 mM EDTA), 5× Denhardt's solution, 0.5% SDS, and 100 μg/ml salmon sperm DNA for 16–20 hr. The filters were washed two times at room temperature in 2× standard saline citrate (SSC)/0.1% SDS, one time at room temperature in 0.5× SSC/0.1% SDS, and one time in 0.5× SSC/0.1% SDS at 55°C and were exposed overnight. Positive plaques were purified through quaternary screens before the insert was PCR amplified using λgt10-specific PCR primers (5′ primer, AGCAAGTTCAGCCTGG-TTAAG; 3′ primer, 5′-TTATGAGTATTTCTTCCAGGG). PCR products were subcloned into pGEM-T (Promega) and partially sequenced with T7 and SP6 primers. This approach isolated a Dwf-A cDNA of 1639 nucleotides and a Dwf-C cDNA of 2185 nucleotides. Both contain an open reading frame of 465 amino acids with a predicted molecular mass of 52 kDa. Subcloning and deletional analysis combined with automated sequencing yielded the nucleotide sequences of Dwf-A and Dwf-C.

Mammalian expression constructs were constructed using *Bam*HI fragments containing full-length cDNAs for Dwf-A and Dwf-C generated by PCR using the following primer sets: Dwf-A 5′ primer, 5′-CGCGGATCCGCGATGAATGTGAC-CAGCTTG; Dwf-A 3′ primer, 5′-CGCGGATCCGCGCAG-AGTTACCAGGTTTGGC; Dwf-C 5′ primer, 5′-CGGGATC-CCGGAATTCCATGACGTCAATGGCCAGC; and Dwf-C 3′ primer, 5′-CGCGGATCCGCGGTAAGGCAAAGAAATT-CC. The resulting PCR products were subcloned into pGEM-T and subsequently into pCMV5 and confirmed by sequencing with a cytomegalovirus promoter-specific sequencing primer: 5′-GCGGTAGGCGTGTACGG-3′.

Northern Analysis. A rat multiple-tissue Northern blot (Clontech) containing 2 μg of poly(A)$^+$ RNA was sequentially probed with the 1.6-kb full-length Dwf-A cDNA followed by the 2.1-kb full-length Dwf-C cDNA. The probes were labeled with [α-^{32}P]dCTP by random priming (Stratagene) and hybridized for 16–20 hr at 42°C in 50% formamide, 5× SSPE, 5× Denhardt's solution, 0.5% SDS, and 100 μg/ml salmon sperm DNA. The filters were washed two times at room temperature in 2× SSC/0.1% SDS and two times in 0.2× SSC/0.1% SDS at 60°C. Autoradiography was at −80°C for 18–24 hr with intensifying screens.

Antibody Production. Bacterial expression constructs for Dwf-A and Dwf-C were constructed using the *Bam*HI fragments from pGEM-T Dwf-A or Dwf-C, respectively, to clone in-frame into pGex 2T, generating glutathione *S*-transferase fusion proteins. The resulting ≈85-kDa fusion proteins were used as antigens for rabbit polyclonal antibody production. Preimmune sera were obtained from each animal before the primary injection of antigen. Both the Dwf-A and Dwf-C antibodies specifically recognize 56-kDa and 52-kDa proteins on Western blots of NMuMg or L6 lysates (data not shown). In addition, transfection of Dwf-A or Dwf-C cDNA into COS cells generates a tight doublet around 52 kDa which cannot account for the two bands seen by Western blotting of endogenous proteins (data not shown). Thus, the 56-kDa protein is most likely immunogenically related to the dwarfins and represents an additional dwarfin family member.

***In Vivo* Phosphorylation of Endogenous Dwarfins.** NMuMg or L6 cells were plated at 1.5 × 10^6 cells per 100-mm dish and allowed to attach overnight. The cells were rinsed once with phosphate-free media (ICN) and then starved in phosphate-free media containing 0.5% dialyzed FBS (GIBCO/BRL) for 1 hr and then labeled in 0.5% dialyzed FBS with 0.5 mCi/ml (1 Ci = 37 GBq) [^{32}P]orthophosphate for 4 hr. TGF-β1 or BMP-2 was added at various concentrations for the indicated length of time at the end of the labeling period. Cells were rinsed twice with ice-cold PBS and lysed in 50 mM Tris (pH 7.5), 100 mM NaCl, 50 mM NaF, 0.5% Nonidet P-40, 1 mM Na_3VO_4, 0.5 mM Na_2MoO_4, 1 mM DTT, 1× protease inhibitors (5 μg/ml antipain, aprotinin, leupeptin, and trypsin inhibitor; 0.5 μg/ml pepstatin), and 1 mM phenylmethylsulfonyl fluoride on ice for 15 min. Lysates were microcentrifuged at 14,000 rpm at 4°C for 15 min. The resulting cell lysates were precleared with protein-A Sepharose for 30 min at 4°C. Lysates were then divided for immunoprecipitation with Dwf-A or Dwf-C antibodies. Preimmune or immune sera (20 λ of unpurified sera) was added with Protein-A sepharose for 4 hr at 4°C. The immunoprecipitates were washed three times with lysis buffer before separation in SDS/8% polyacrylamide followed by autoradiography at room temperature.

Transient Growth Arrest Assay. The following procedure was adapted from DeGregori and coworkers (18). L6 cells were plated in six-well trays on poly-L-lysine-coated coverslips at 10^5 cells per well and allowed to attach overnight. CellFectin (GIBCO/BRL) was used to transfect the cells with 2 μg of pCMV LacZ and 8 μg of vector alone, Dwf-A, or Dwf-C for 10 hr in DMEM. The cells were incubated in 10% FBS for 36 hr before immunohistochemical staining. Media containing 20 μM bromodeoxyuridine (BrdU) was added for the last 20 hr to metabolically label dividing cells. The cells were fixed in 4% paraformaldehyde in PBS, permeabilized with MeOH/acetone, and stained with a 1:500 dilution of rabbit anti-β-galactosidase antibody (5 Prime–3 Prime, Inc.) in 1% BSA/PBS for 60 min, followed by a fluorescein isothiocyanate-conjugated goat anti-rabbit secondary antibody (Boehringer Mannheim) at 1:500 in 1% BSA/PBS to stain transfected cells. After a second fixation and 2 M HCl permeabilization for 45 min, the cells were stained with a mouse anti-BrdU antibody (Zymed) at 1:3 in 1% BSA/PBS followed by a goat anti-mouse rhodamine-conjugated secondary antibody (Pierce) at 1:100 in 1% BSA/PBS. Finally, 4 μg/ml Hoeschst 33342 in PBS was used to counter-stain the DNA. The coverslips were mounted in 1,4-diazabicyclo[2.2.2]octane and analyzed by fluorescence microscopy.

RESULTS

Cloning of Dwf-A and Dwf-C. Full-length cDNAs for Dwf-A and Dwf-C were isolated by low stringency hybridization of a 8.5-day mouse embryonic library as described in *Materials and Methods*. The amino acid sequences of Dwf-A and Dwf-C are 95% homologous and 90% identical. Both contain the characteristic dwarfin homology domain 1 (DH1) and dwarfin homology domain 2 (DH2) motifs separated by a proline-rich linker region (Fig. 1*A*; ref. 15). Dwf-A and Dwf-C are about 80% homologous to the *Drosophila* dwarfin, MAD (Fig. 1*A*; ref. 14), and the *C. elegans* dwarfin, SMA-2 (Fig. 1*A*; ref. 15). Dwf-A and Dwf-C are distantly related to the only other known mammalian dwarfin, DPC4 (60% homologous, 40% identity; ref. 17), and thus likely represent a distinct family of mammalian dwarfins (Fig. 1*B*). Northern analysis showed that both genes are ubiquitously expressed (Fig. 1*C*). The mRNA message of Dwf-A is ≈3.5 kb, whereas Dwf-C has two messages of ≈8 and ≈3 kb.

TGF-β Induces Phosphorylation of Endogenous Dwarfins. We chose two cell lines, NMuMg (19) and L6, which are potently growth inhibited by TGF-β, as model systems to study endogenous dwarfin phosphorylation. Antibodies against Dwf-A or Dwf-C were used to determine if the levels or phosphorylation state of endogenous dwarfins were modulated by TGF-β. The expression levels of dwarfins were unchanged at any time within 24 hr after TGF-β treatment as determined by Western blot analysis (data not shown). However, TGF-β induced rapid, but transient, phosphorylation of Dwf-A, Dwf-C, and a 56-kDa immunogenically related protein (Fig. 2*A*).

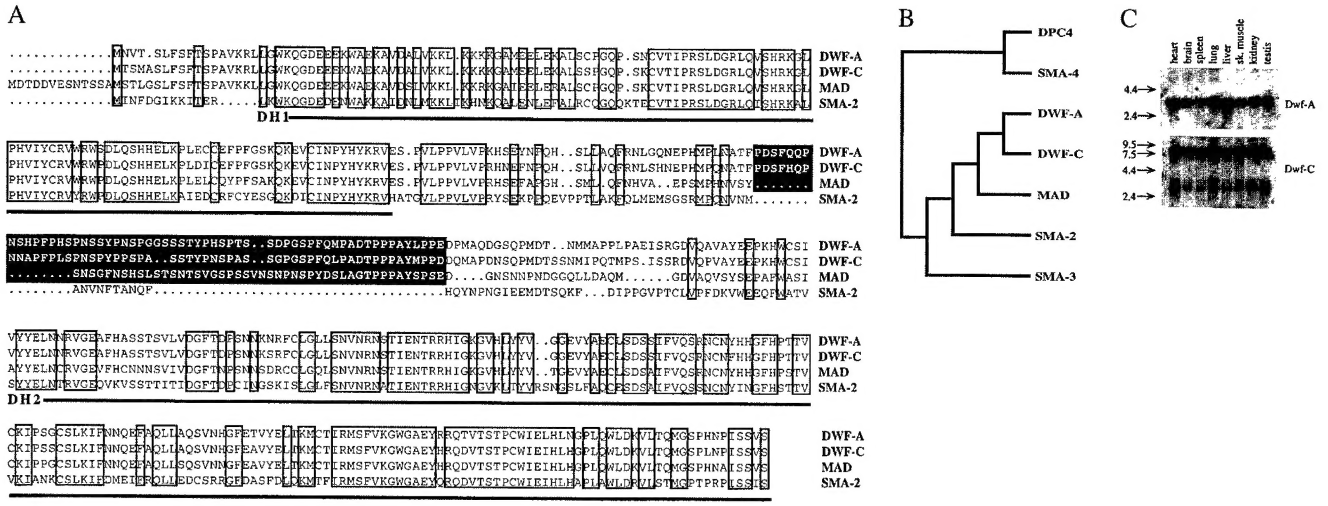

FIG. 1. Predicted amino acid sequence and expression pattern of Dwf-A and Dwf-C. (*A*) Alignment of Dwf-A and Dwf-C with *Drosophila* MAD and *C. elegans* SMA-2. The GenBank accession numbers for Dwf-A and Dwf-C are U58992 and U58993, respectively. The two highly conserved domains, DH1 and DH2, are underlined. The serine/threonine-rich insert in the mammalian dwarfins is highlighted. (*B*) Dendrogram analysis of the dwarfin protein family. (*C*) Northern analysis of Dwf-A and Dwf-C. A rat multiple tissue Northern blot was sequentially probed with the 1.6-kb full-length Dwf-A cDNA followed by the 2.1-kb full-length Dwf-C cDNA.

Phosphorylation of Dwf-A and Dwf-C was induced within 15 min and peaked at 2.4-fold and 4-fold by 60 min, respectively (Fig. 2*B*). The difference in the extent of phosphorylation of Dwf-A and Dwf-C is due to the lack of basal phosphorylation of Dwf-C in the absence of TGF-β treatment (Fig. 2*A*). Phosphorylation of the 56-kDa protein in both αDwf-A and αDwf-C immunoprecipitates was also induced within 15 min and peaked at 3.2-fold by 60 min (Fig. 2*B*). By 4 hr the phosphorylation state of all three proteins had returned to nearly basal levels. The induced phosphorylation of Dwf-A, Dwf-C, and the 56-kDa protein was primarily on serine residues ($\approx$90%) with minor threonine phosphorylation ($\approx$10%) as determined by phosphoamino acid analysis (data not shown).

Immunoprecipitation of Dwf-A and Dwf-C from L6 cells gave nearly identical results as those from NMuMg cells (Fig. 2 *C* and *D*). Dwf-A and the 56-kDa protein were phosphorylated to a similar extent and with similar kinetics in both cell lines. Dwf-C was phosphorylated with the same kinetics as in NMuMg cells, but with a reduced extent due to increased basal phophorylation in the absence of TGF-β in L6 cells (Fig. 2*C*).

To ensure that TGF-β was capable of inducing phosphorylation of the dwarfins at more physiological concentrations, we determined the dose dependence of dwarfin phosphorylation in NMuMg cells. Dwf-A, Dwf-C, and the 56-kDa protein were all inducibly phosphorylated by 10 pM TGF-β (Fig. 3), a concentration capable of eliciting the various biological effects of TGF-β.

BMP-2 Induces Phosphorylation of Endogenous Dwarfins. TGF-β-like ligands from *Drosophila* and *C. elegans* are most closely related to mammalian BMPs (16, 20). BMPs have been shown to elicit multiple effects, including inhibition of cellular proliferation, on many different cell types (21). Like TGF-β, BMP-2 has been shown to initiate its signaling cascade by

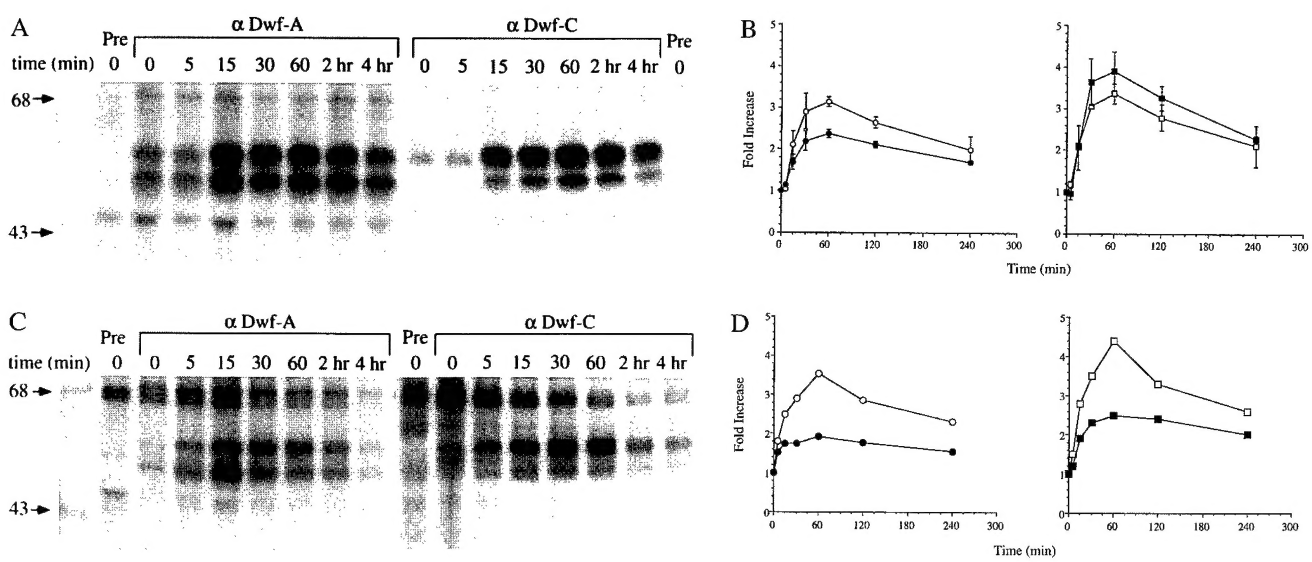

FIG. 2. TGF-β-induced phosphorylation of endogenous dwarfins. Immunoprecipitation with Dwf-A (*Left*) or Dwf-C (*Right*) antibodies was performed on NMuMg (*A*) or L6 (*C*) cells treated for the indicated length of time with 500 pM (7.5 ng/ml) TGF-β1. Quantitation of TGF-β induced dwarfin phosphorylation in NMuMg (*B*) or L6 (*D*) cells. The 52-kDa Dwf-A (●) and Dwf-C (■) bands were quantitated using a PhosphoImager. The 56-kDa protein in αDwf-A (○) or αDwf-C (□) immunoprecipitates was also quantitated. The level of phosphorylation was standardized using the 68-kDa background band and two additional background bands.

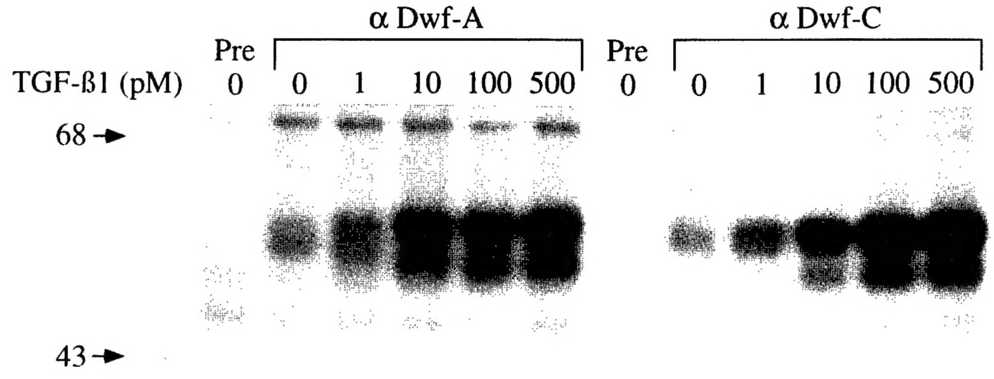

FIG. 3. TGF-β phosphorylation of endogenous dwarfins is dose-dependent. Dwf-A and Dwf-C immunoprecipitation from NMuMg cells was performed as in Fig. 2 except various doses of TGF-β1 were added for 1 hr.

binding to a heteromeric complex of transmembrane serine-threonine kinase receptors at the cell surface (22). However, the similarity between the cytoplasmic pathways that lead to the biological effects of TGF-β and BMP-2 is unknown. The NMuMg cell line is potently inhibited by BMP-2, affording us the opportunity to determine if dwarfin phosphorylation is induced by BMP-2. As shown in Fig. 4 *A* and *B*, phosphorylation of the 56-kDa protein is induced by BMP-2 within 15 min and peaks at 2.5-fold in 60 min. Interestingly, Dwf-A and Dwf-C, which are inducibly phosphorylated in response to TGF-β, are not phosphorylated in BMP-2-treated NMuMg cells (Fig. 4*A*).

Dwf-A and Dwf-C Are Implicated in Cell Growth Regulation. Attempts to establish stable cell lines constitutively overexpressing either Dwf-A in L6 cells or Dwf-C in NMuMg cells were unsuccessful. This result was not unexpected because the dwarfins are suspected to have growth-suppressive effects based on the tumor suppressor activity of DPC4 (17). Consequently, we used a modified transient growth assay (18) to assess the ability of Dwf-A or Dwf-C to cause a growth arrest when transiently transfected into L6 cells. Constitutive overexpression of Dwf-A or Dwf-C caused 30–40% growth inhibition compared with 10% for control vector transfectants (Fig. 5). Therefore, Dwf-A and Dwf-C, like DPC4, exhibit growth-inhibitory properties, implicating these dwarfin proteins in cell growth regulation.

DISCUSSION

Identification of downstream effectors for TGF-β or TGF-β superfamily members has proven elusive. Mutagenesis studies in mammalian cells have yielded only receptor mutants (23, 24), which suggests the existence of redundant pathways downstream of the receptors. Fortunately, TGF-β-like pathways exist in genetically tractable organisms to allow the use of genetics to identify components of these signaling pathways. Many of these components (e.g., receptors, accessory molecules, and ligands) have been shown to have homologous counterparts in vertebrate systems. Consequently, we have studied two mammalian homologs of MAD and SMA-2 as potential downstream effectors of the TGF-β signaling pathway. We provide biochemical evidence that the dwarfin family of proteins is involved in TGF-β and BMP-2 signaling pathways in mammalian systems. Furthermore, results from a modified transient growth assay and preliminary studies with potentially dominant negative forms of Dwf-A (unpublished data) strongly implicate a role for the dwarfins as mediators of the TGF-β growth-regulatory signal.

Although the dwarfins do not contain any known catalytic motifs, their DH1 and DH2 domains are reminiscent of Src homology 2 and 3 domains, which in a variety of signaling pathways modulate protein–protein interactions based on tyrosine phosphorylation and proline-rich sequences, respectively (25). TGF-β and BMP-2-induced phosphorylation of the dwarfins may regulate protein–protein interactions in an analogous fashion for TGF-β superfamily signaling cascades. Recently, 14-3-3 proteins have been shown to be specific phosphoserine-binding proteins that are critical for the activation of signaling proteins (26). This suggests a novel role for serine–threonine phosphorylation in the assembly of protein–protein complexes required to transduce certain intracellular signals. Serine–threonine phosphorylation of the dwarfins may regulate their ability to serve as adaptor molecules for other effectors in the TGF-β pathway or regulate their ability to specifically bind other intracellular proteins. These protein–protein interactions may result in altered subcellular distribution of the dwarfins. Preliminary immunofluorescence studies in NMuMg cells indicate that the dwarfins are predominantly

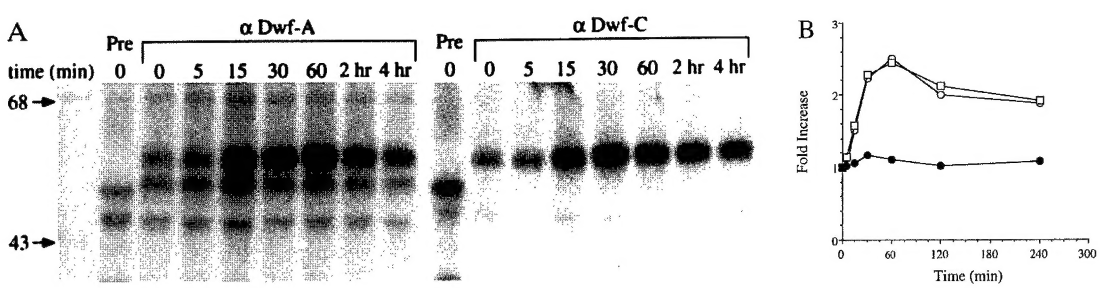

FIG. 4. BMP-2-induced phosphorylation of endogenous dwarfins. (*A*) Immunoprecipitation with Dwf-A (*Left*) or Dwf-C (*Right*) antibodies was performed on NMuMg cells treated for the indicated length of time with 100 ng/ml BMP-2. (*B*) Quantitation of BMP-2-induced dwarfin phosphorylation in NMuMg cells. The 52-kDa Dwf-A band (●) and the 56-kDa protein in αDwf-A (○) or αDwf-C (□) immunoprecipitates were quantitated. The 52-kDa Dwf-C band that is not basally phosphorylated in NMuMg cells is undetectable after BMP-2 treatment. The level of phosphorylation was standardized using the 68-kDa background band and two additional background bands.

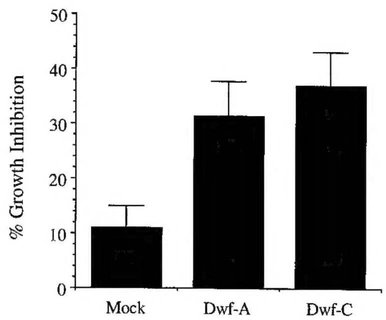

FIG. 5. Dwf-A and Dwf-C cause a growth arrest in L6 cells. L6 cells were transfected and immunohistochemically stained as described in *Materials and Methods*. All the transfected cells (fluorescein isothiocyanate stained) on each coverslip were scored for BrdU incorporation (rhodamine stained). All (100%) of the nontransfected cells incorporate BrdU during the labeling period; therefore, the percentage of transfected cells that are BrdU-negative represents the percent growth inhibition caused by transfection of the cDNA. Data shown are the mean ± SD of at least three experiments.

localized in the cytoplasm (unpublished data). Although TGF-β or BMP-2 treatment does not appear to cause a significant change in the subcellular distribution of the dwarfins, it is possible that a minor proportion of dwarfins that become phosphorylated accumulate at the membrane or translocate to the nucleus to fulfill their biological function. Indeed, a human homolog of Dwf-A, MADR1, has recently been shown to be inducibly phosphorylated and to translocate to the nucleus after BMP-2 treatment (27). Intriguingly, phosphorylation of MADR1 appears to be BMP-2-specific in their system, since neither TGF-β nor activin induces MADR1 phosphorylation. This apparent discrepancy may be due to differences in experimental systems, overexpression of epitope-tagged MADR1 instead of endogenous dwarfins, or may represent cell-type specific differences in the pathways used by TGF-β superfamily ligands. Clarification of this issue requires further study.

Our initial attempt to study TGF-β's ability to modulate the phosphorylation state of Dwf-A and Dwf-C involved transfection of hemagglutinin epitope-tagged cDNAs into COS or mink lung epithelial cells. Although both proteins are phosphorylated in these systems, the high level of constitutive phosphorylation precluded detection of TGF-β induced changes in Dwf-A or Dwf-C phosphorylation (unpublished data). Interestingly, the *C. elegans* dwarfins, SMA-2 and SMA-3, are not phosphorylated when overexpressed in COS cells. Therefore, the observed phosphorylation is likely a result of phosphorylation by an associated kinase that is unable to recognize SMA-2 or SMA-3 as substrates. The unique serine-threonine-rich insert in the linker region of Dwf-A and Dwf-C (Fig. 1*A*) may play a role in either kinase recognition or as targets of phosphorylation by the associated kinase. Preliminary results indicate that neither Dwf-A nor Dwf-C are substrates of the type I or type II TGF-β receptor kinases (unpublished data), implicating an as yet unidentified kinase in phosphorylation of the dwarfins.

The differential phosphorylation of endogenous dwarfins in response to TGF-β and BMP-2 suggests that the specific dwarfins used by TGF-β superfamily members may vary. In support of this notion, two related *Xenopus* dwarfins (Xmad1 and Xmad2) have been shown to mediate either BMP-2/BMP-4 signals or Vg1/activin/nodal signals, respectively (28). Thus, Dwf-A and Dwf-C may represent TGF-β-specific dwarfins, whereas the 56-kDa dwarfin is shared by TGF-β and BMP-2. The differential response of these proteins to TGF-β and BMP-2 will facilitate elucidation of the differences in the intracellular pathways used by these two members of the TGF-β superfamily.

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